TEMPERATURE AND TOXIC ACTION.

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(WITH THIRTY-THREE CHARTS)

THE purpose of these experiments, the results of which are presented in this paper, was to determine what might be the modifying effect of temperature on the toxic properties of certain chemicals as shown by the effect of these substances on germination and growth in certain fungi. Since chemical processes as well as plant activities are influenced by temperature, it was thought that additional knowledge in regard to the nature of the physiological action of poisons might be obtained by comparing their effects at the optimum temperature for germination and growth of the plant with results secured under otherwise similar conditions, but at temperatures below and above that which is most favorable for the development of the particular plant.

So far as the writer has been able to learn, the problem of toxic action has never been carefully investigated from this standpoint. It is well known that temperature is an important factor in the processes of plant and animal life, and that changes in temperature may often serve as a stimulus to reproduction, germination, and development. It has also been shown that the response of an organism to certain stimuli may vary with the temperature, and some data have been reported which indicate that this is true when the stimulus is of a chemical nature. THIELE (1) found that the maximum temperature for the growth of *Penicillium glaucum* on grape sugar lies at 31° C., on glycerin at 36° C., on salts of formic acid at 35° C. NÄGELI (2) reported that bacteria were killed at 30 or 110° C. according to the character of the nutrient medium, but his conclusions seem to be based upon results obtained from impure cultures. HEIDER (3) found that the toxic action of certain chemicals upon the spores of *Bacillus anthracis* increases with a rise of temperature. PASTEUR (4) found that bacteria were more resistant to heat in alkaline than in acid milk; but COHN (5) and BREFELD (6) observed no such increased resistance in alkaline solutions. RICHET (7) has reported that with

various poisons the toxic dose diminishes in amount with the elevation of the temperature of the body. MATHEWS (**8**) found that a small rise in room temperature increased the toxic action of certain salts upon the eggs of the fish *Fundulus heteroclitus*, but no data in regard to the extent of the injury were reported.

Considerable work has been done in recent years on the effect of toxic agents upon the germination and development of fungi. CLARK (**9**) determined the concentration of various chemical solutions necessary to produce injury, inhibition, and death in certain fungi. He found that a solution of $n/4$ HNO_3 killed the spores of *Sterigmatocystis nigra* within forty-eight hours, that $n/8$ to $n/16$ solutions of the same acid produced total inhibition of the spores, and that $n/32$ gave great injury to the fungus. *Botrytis vulgaris* spores were killed by $n/16$, and the plant was greatly injured by $n/32$ HNO_3. With *Penicillium glaucum*, $n/4$ HNO_3 killed the spores, $n/8$ and $n/16$ totally inhibited germination, and $n/32$ gave decided injury. H_2SO_4 gave similar results, but a concentration of $n/2$ was required to kill the spores of Sterigmatocystis and Penicillium. With $CuSO_4$, $n/4$ killed the spores of Sterigmatocystis, $n/8$ to $n/16$ gave total inhibition, and $n/32$ to $n/64$ caused decided injury. Botrytis spores were killed by $n/16$ $CuSO_4$, inhibited by $n/32$, and the plant greatly injured by $n/64$. The spores of Penicillium were killed by $2n$ and inhibited by n to $n/64$, while decided injury resulted from $n/128$. DUGGAR (**10**) has reported upon special factors that influence the germination of fungous spores, and Miss FERGUSON (**11**) has given some of the conditions for germination in various basidiomycetous fungi. These recent papers have only an indirect bearing upon the work that follows, but have been very useful in the suggestion of methods for the solution of the problem.

METHODS.

The effect of the various toxic solutions at the different temperatures was observed by means of the ordinary Van Tieghem cells. The manner of constructing and the method of using these have been fully described by CLARK (**9**) and DUGGAR (**10**).

These cells were never used a second time without being taken apart and thoroughly cleaned. In cleaning, the cells were boiled for twenty or thirty minutes, first in an alkali, then in an acid, and

finally in distilled water. They were dried from alcohol and made up in the usual manner. The covers were treated as the cells, except that in each instance they were heated for a longer time, and that they were given one or two final boilings in redistilled water. All flasks, vials, etc., used in these experiments were cleaned with alkali, acid, and distilled water by boiling, as described for the cells.

As a culture medium several vegetable decoctions were tried. It was found that the five fungi used in these experiments grew well upon decoctions made from onions, beets, tomatoes, grapes, parsnips, beans, mushrooms, and sugar beets. Several series of experiments were made with tomato decoction as a medium, but it was found that a sugar beet solution gave less precipitate in the presence of $CuSO_4$ and was in general more satisfactory for the work. In all the experiments reported in this paper beet decoction was used as the nutrient medium. In making the infusion 600 grams of beets were used for every liter of water. At the time of using, the decoction was diluted, by the addition of the toxic solution and water, to one-half of its former nutrient value.

The toxic agents used were HNO_3, H_2SO_4, and $CuSO_4 + 5H_2O$. The chemicals were of the highest quality that could be obtained and the acid solutions were standardized before using. It is a well-known fact that strong concentrations of $CuSO_4$ precipitate proteids. In solutions at ordinary temperatures in which both are present, this precipitation continues for a long time, thus continually changing the nature of the liquid. Therefore, as it was necessary to make experiments at considerable intervals of time, the toxic agent was not added to the beet decoction until the time of its use in cultures. Stock solutions of the chemicals were made in water that had been carefully redistilled from glass in the presence of an oxidizing agent. Normal or one-half normal solutions were made and these stored in flasks provided with closely fitting rubber stoppers. By means of a series of graduated vials, these stock solutions were diluted and mixed with the beet decoction at the time of using.

The fungi used were *Botrytis vulgaris*, *Monilia fructigena*, *Sterigmatocystis nigra*, *Mucor Mucedo*, and *Penicillium glaucum*. The first two may be and usually are parasitic, and have an optimum temperature that is comparatively low; the last three are saprophytic

and grow well at temperatures considerably above the optimum for the first two. It was thought by this selection to obtain more interesting results than with forms more closely related physiologically. Only pure cultures were used. In the test tube cultures from which the spores were obtained for use, the fungi were grown upon cylinders of potato or beet. In either case the liquid in the tubes was a decoction of sugar beets. Other nutrient substances were tried for the test tube cultures, but these usually produced modifications in the growth of the fungi and it was not found advisable to use spores produced on different media in the course of a series of experiments, the results of which were to be compared. The spores used were always taken from cultures that were twelve to sixteen days old. The desired temperatures were secured by means of incubators and a refrigerator.

CLARK has pointed out certain sources of error for Van Tieghem cell cultures exposed to ordinary temperatures; but the placing of cells, made up under ordinary laboratory conditions, at temperatures ranging from 5° to 30° C., gave additional opportunity for error. The cells were not entirely closed until they had been left for several minutes in the temperature at which they were to remain. This gave opportunity for adjustment of air pressure in the cell, but it did not in all cases prevent the condensation of water vapor upon the cover glass. The small drops of water thus formed not only increased the evaporating surface but also modified the vapor pressure in the cell. The small water drops adjacent to the hanging ones of the nutrient solution seemed to sometimes unite with them, thus changing both their size and concentration. When the cultures were made in the dry air of a furnace heated room no difficulty was experienced, but cells made upon sultry days, or when the air of the culture room was humid from any cause, gave a visible condensation when placed at low temperatures. Even with the greatest precaution this difficulty was not entirely overcome.

It was found difficult to examine the cultures placed at various temperatures without interfering with the structure and condition of the cells. Examinations were made at temperatures as near as possible to those at which the fungi were growing, and results obtained from damaged cells were rejected. All cultures were observed every

twenty-four hours and notes taken of percentage of germination, length of germ tube, fruiting, and any peculiarities in germination or development. More frequent observations would have been of interest, but they were not made on account of the increased source of error that would have been thus introduced. Any sources of error that were not otherwise provided for were guarded against by always making duplicate cultures. The experiments with the three chemicals were always made at different times, and as control cultures were made in every case, the growth of each fungus in a nutrient medium at a particular temperature was tested six times.

The vitality of spores that had been subjected to the action of an inhibiting toxic agent was tested by transfer to a nutrient non-toxic medium. An attempt was made to accomplish this transfer by removing the drop of the toxic solution with sterilized filter paper and replacing it by a drop of beet decoction. This method left some part of the former solution as well as any precipitate that had been formed adhering to the cover glass, and was therefore adandoned. All transfers that are concerned in the following data were made by means of a sterilized platinum needle. The spores were in every case transferred to a drop of beet decoction on a clean cover glass. The medium used in the bottom of the cells was in this, as well as in all other cases, the same as that of the hanging drop. It is quite evident that the above method of transferring did not prevent a small amount of the toxic solution being carried into the new drop by the spores and the needle, but the results obtained indicated that this small per cent. of the toxic agent either served as a very slight stimulus to germination and growth or exerted no appreciable influence.

Early in the work it was seen that results obtained from the exposure of the fungous spores to the toxic agent must be considered entirely apart from the data secured in cases where the mycelium was acted upon by the toxic solution. Therefore, when a particular toxic solution gave no germination at one temperature, but did at others, the ungerminated spores were in no case transferred; *i. e.*, transfers were made only with those solutions that gave no germination even at the optimum temperature at the end of the given time.

By a series of preliminary experiments strengths of toxic solution

and time of exposure were determined, such as would give the greatest contrast in the results obtained at the various temperatures.

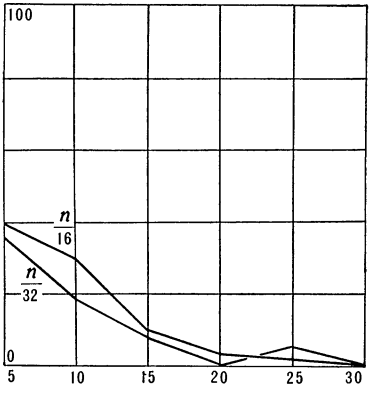
DATA AND DISCUSSION.

In order to put the results obtained in a form as concise as possible, charts 1*a*–12*c* have been prepared, and the greater part of the data obtained is expressed in these by means of curves.

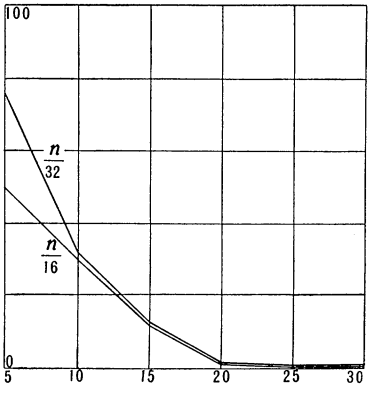
In charts 1*a* to 10*c* inclusive, the abscissae indicate the temperatures at which the fungus was kept in culture, and the ordinates show the per cent. of germination at these temperatures. All the points indicating per cent. of germination at the various temperatures for a particular toxic solution are joined by solid or broken lines; the strength of the toxic solution used is shown by the fraction placed on or near the particular curve. For a further illustration of the meaning of these charts, the curves in chart 2*a* may be considered. These represent the data secured by using $CuSO_4$ with Monilia. With an $n/16$ solution no germination was obtained at 25° and 30°; but at 15°, 12 per cent. of the spores germinated; at 10°, 30 per cent.; and at 5°, 49 per cent.

In charts 1*a* to 5*c* inclusive the results were obtained by exposing the spores for twenty-four hours at the various temperatures in the toxic solution indicated and then transferring them as previously described. The charts are based entirely upon the data secured on the first and second days after transferring. The solid lines indicate the total germination at the end of the second day. The broken lines show the per cent. of germination twenty-four hours after transferring. Where the record of germination was the same for the two days only the solid line is used. It will be noticed that only in a very few instances did spores germinate on the second day.

It is readily seen that in most cases the deleterious action of the toxic agents increased very rapidly with the rise in temperature. A comparison of the charts for the various fungi indicates that there are some differences in reactions worthy of special note. Thus, there is a marked drop between 5° and 10° in the germination curves for Botrytis and Monilia, but for no other fungus. With Penicillium the fall comes either between 10° and 15° or between 15° and 20°, while with Mucor and Sterigmatocystis the downward curves begin

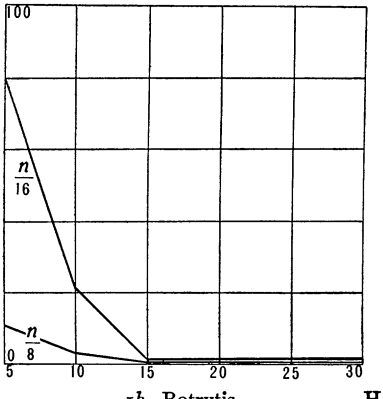


1*a*, Botrytis.

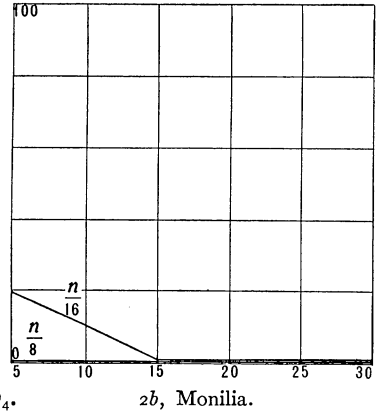


2*a*, Monilia.

$CuSO_4$.

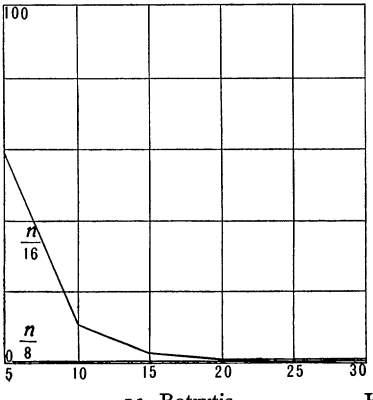


1*b*, Botrytis.

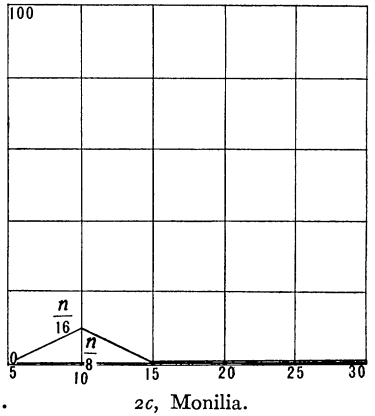


2*b*, Monilia.

H_2SO_4.

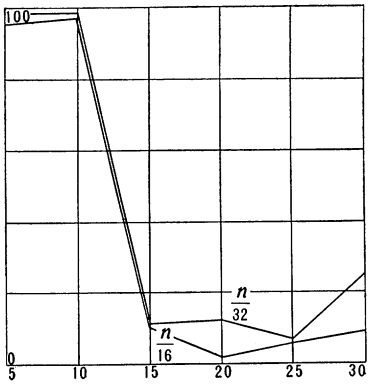


1*c*, Botrytis.

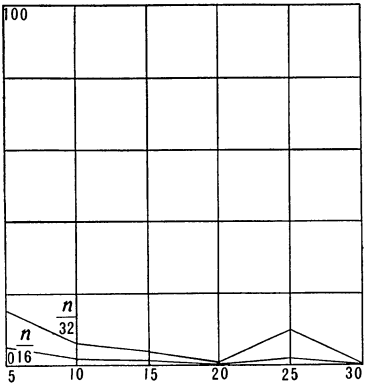


2*c*, Monilia.

HNO_3.

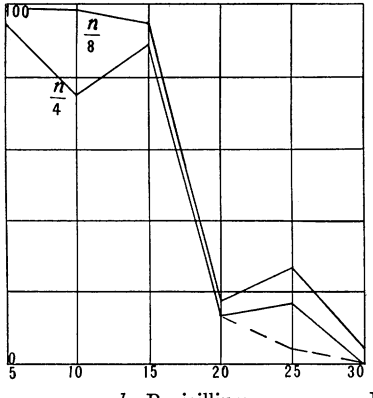


3*a*, Penicillium.

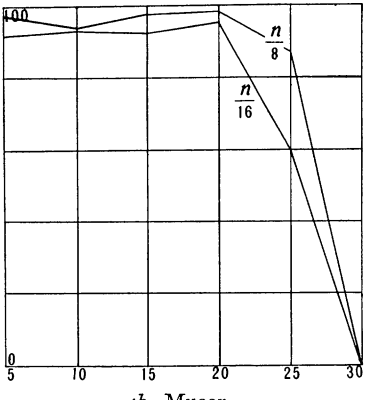


4*a*, Mucor.

$CuSO_4$.

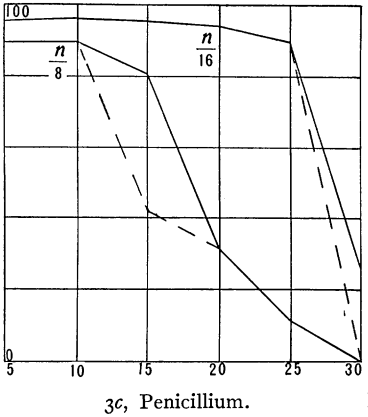


3*b*, Penicillium.

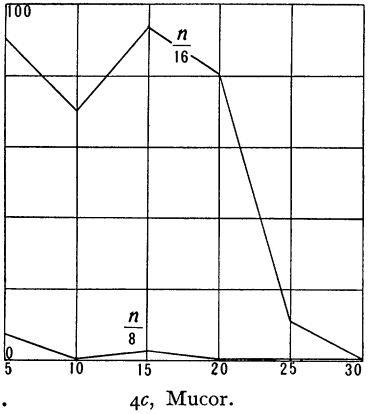


4*b*, Mucor.

H_2SO_4.

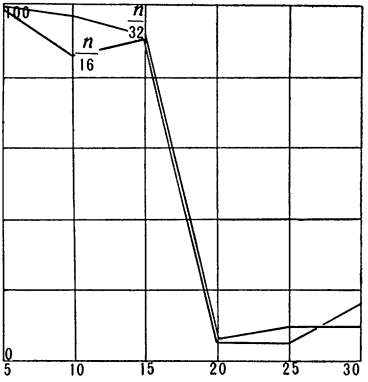


3*c*, Penicillium.

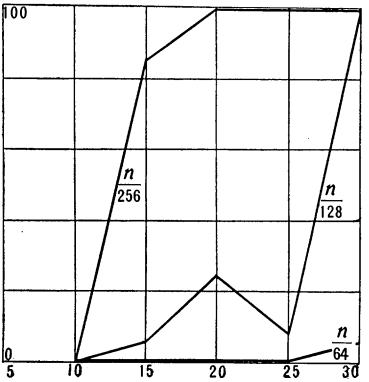


4*c*, Mucor.

HNO_3.

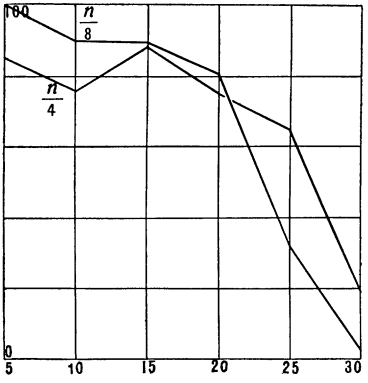


5*a*, Sterigmatocystis.

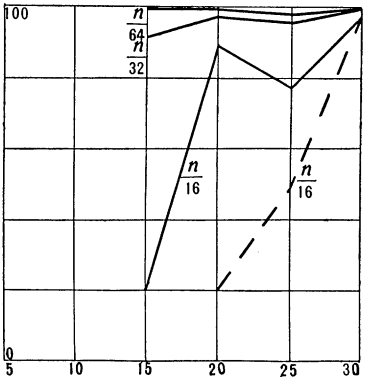


6*a*, Sterigmatocystis.

$CuSO_4$.

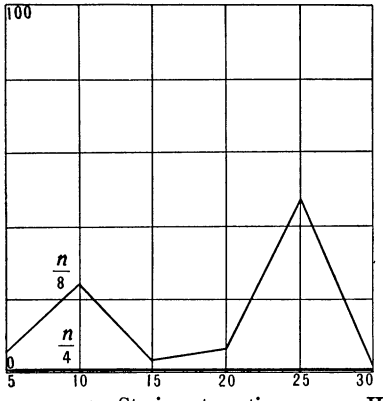


5*b*, Sterigmatocystis.

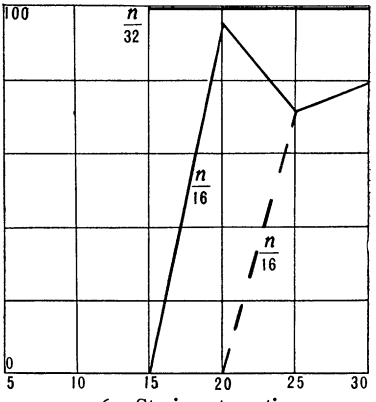


6*b*, Sterigmatocystis.

H_2SO_4.

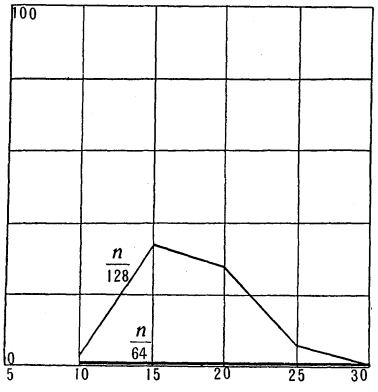


5*c*, Sterigmatocystis.

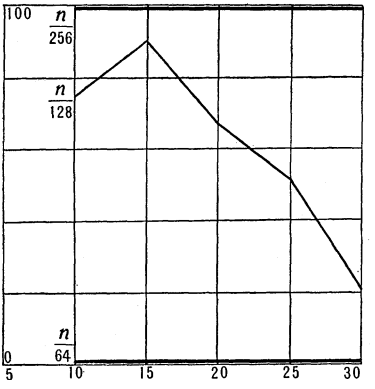


6*c*, Sterigmatocystis.

HNO_3.

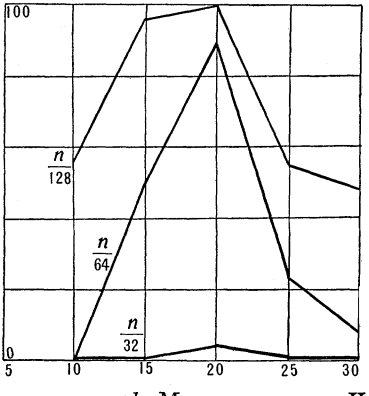


7*a*, Mucor.

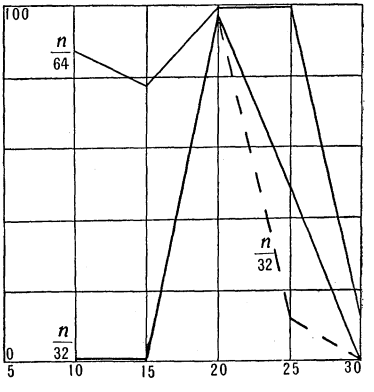


8*a*, Penicillium.

$CuSO_4$.

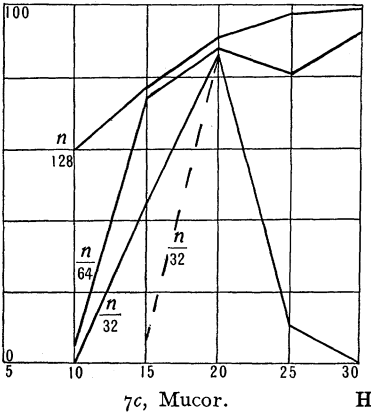


7*b*, Mucor.

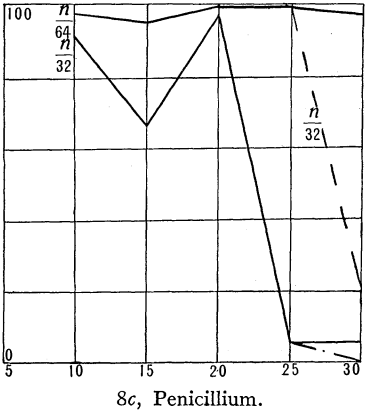


8*b*, Penicillium.

H_2SO_4.

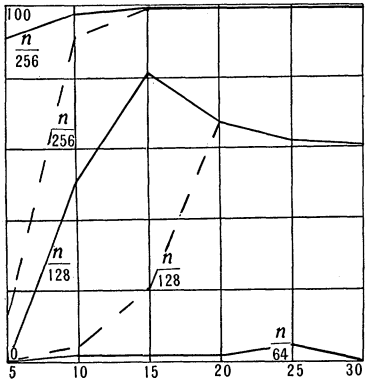


7*c*, Mucor.



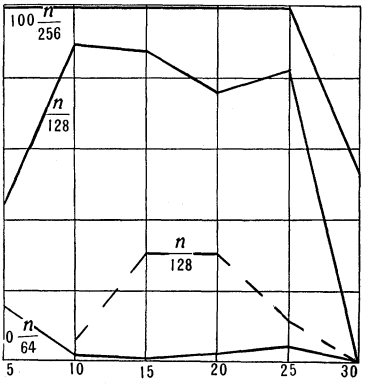
8*c*, Penicillium.

HNO_3.

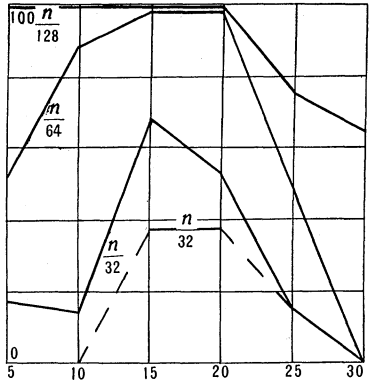


9*a*, Botrytis.

$CuSO_4$.

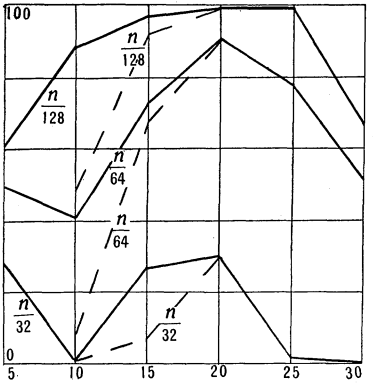


10*a*, Monilia.

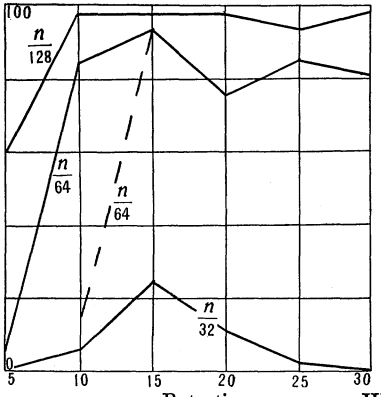


9*b*, Botrytis.

H_2SO_4.

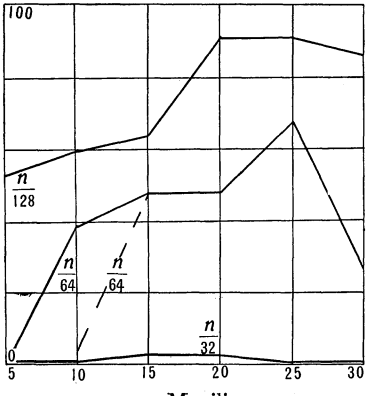


10*b*, Monilia.



9*c*, Botrytis.

HNO_3.



10*c*, Monilia.

at 15° or 20°. This variation may be the result of using solutions that were, without regard to temperature, more injurious to some of the fungi than to others; but strong concentrations, such as $n/4$ and $n/8$ HNO_3, when used with Mucor and Sterigmatocystis, have not given the rise in the curves from 10° to 5° that has been repeatedly obtained with Botrytis and Monilia. It should be noted that these last two fungi are not only the ones that are most greatly injured by the toxic agents, but also are those that require the least stimulus for germination. DUGGAR (**10**) found that both Botrytis and Monilia germinated readily in distilled water, but that Sterigmatocystis and Penicillium did not germinate.

There is also a remarkable agreement in the minimum temperature for the germination of a particular fungus under certain conditions and the location of the fall in its germination curve. Botrytis and Monilia not only show the greatest increase in toxic effect on passing from 5° to 10°, but they are also the only ones that had germinated in the control cultures at 10° by the end of one day. With Penicillium the $CuSO_4$ and HNO_3 curves show a tendency to drop between 10° and 15°, while in the H_2SO_4 curves the fall comes beyond 15°. Along with these data should be noted the fact that the control with the H_2SO_4 cultures gave no germination in one day, while those with the HNO_3 and $CuSO_4$ cultures had germinated in this time. This variation in the controls was probably due to a slight change in the temperature of the refrigerator, together with the fact that 15° approaches the lower limit of temperature for obtaining the germination of Penicillium in one day (WIESNER **13**). Mucor gave no germination the first day in the controls at 15°, with the exception of about 14 per cent. with the $CuSO_4$ series, and Sterigmatocystis in no instance germinated in one day at this temperature. As has been already mentioned, the curves for these fungi fall between 15° and 20° or at a higher temperature. From these facts it is seen that the spores exposed to a harmful agent and at the same time inhibited by cold have not been greatly injured.

In the cultures from which the data in charts *6a* to *10c* inclusive were obtained, the spores were in no instance transferred. The cells in the $CuSO_4$ series remained at the temperature indicated for four days; those with the acids were removed at the end of two days.

In the former six daily observations were made, in the latter only four. Control cultures of spores in beet decoction were kept with the toxic cultures at all times. These controls were subjected to the various temperatures for the same length of time as the other cultures. The per cent. indicated in these charts do not in every instance represent the actual germination, but were in all cases obtained by dividing the per cent. of germination in the particular culture by that in the control at the same temperature. It was found more difficult to represent in graphical manner the results obtained from these experiments, since the per cent. of germination did not always seem to agree with the extent of the injury. The solid lines show the germination at the time of the final removal from the given temperature. The per cent. of germination at the end of twenty-four hours is indicated by the broken lines. These unite with the solid lines as they approach the optimum temperatures. Where no broken line is given the germination was the same at the end of the first day as at the time of removal from the special temperature. The results obtained at temperatures at which the controls had not germinated were omitted from the curves. This accounts for the fact that a number of the curves are not extended to the lower temperatures.

In all instances the injurious effects were least at the optimum for the fungus. This optimum was determined by the germination and development in the controls. The harmful effects were shown by decreased germination as indicated in the charts and by abnormal development. The toxic solutions that gave but partial germination at the optimum for the fungus usually gave only abnormal development above and below that optimum. Thus, Botrytis in $n/32$ HNO_3 gave mycelial development approaching the normal only at 15° and 20°; Penicillium in $n/128$ $CuSO_4$ gave medium growth at 20° but at no other temperature, at 30° the germ tubes seldom became more than a few spore diameters in length even after removal to a more favorable temperature and many spores swelled without germinating. Sterigmatocystis has its optimum above 25° and it is the only fungus in which the injurious effects decreased above that temperature. Both Mucor and Sterigmatocystis germinated and grew well at 35°, the other three fungi gave little or no germination at that temperature.

The charts do not show the results obtained at low temperatures, but in every instance cultures were placed at 5° and 10°. Spores kept for two days in a particular toxic solution at a temperature so cold that it inhibited their germination gave, upon removal to the room temperature, a germination and development that was but little inferior to that obtained from the fresh spores under like conditions of medium and temperature. Spores inhibited at a temperature that did not prevent germination were more greatly injured. Spores of Mucor gave fair growth in $n/32$ H_2SO_4 and $n/32$ HNO_3 after removal from 5°, but after removal from 10° did not germinate. Sterigmatocystis spores in $n/16$ HNO_3 grew almost as well after removal from 10° as from 5°, but in the cultures removed from 15° (a temperature not inhibiting germination in the control) no germination was obtained.

A comparison of the curves obtained with the different chemicals shows that those for weak concentrations of HNO_3 do not drop so rapidly at high temperatures as the curves for weak solutions of the other toxic agents.

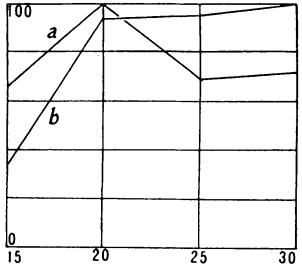
In order to obtain additional information in regard to the significance of the results secured with the cells, a series of flask cultures was made. In every instance 25^{cc} of the given solution were placed in a 100^{cc} flask. These flasks were sterilized after the introduction of the solution. The effect of the toxic agent was determined by taking the dry weight of the fungous growth at the end of the given time. With the exception of $n/64$ $CuSO_4$, duplicate cultures were made and the average weight used in estimating the effect. Flask cultures were made of Mucor, Penicillium, and Sterigmatocystis. The results obtained from cell cultures were used as a basis in determining what strengths of the toxic agents should be used in the flasks. So much greater concentration was required to give injury in flask cultures than in cells that no definite results were obtained with Mucor and Penicillium. Also it was found that Penicillium would not grow in flask cultures placed at 30° C., a temperature at which the fungus grew well in the control cell cultures.

In the series with Sterigmatocystis the air in the incubator was dry, while that in the refrigerator was kept damp by the melting ice. It was feared that the evaporation from the flasks at 25° and 30°

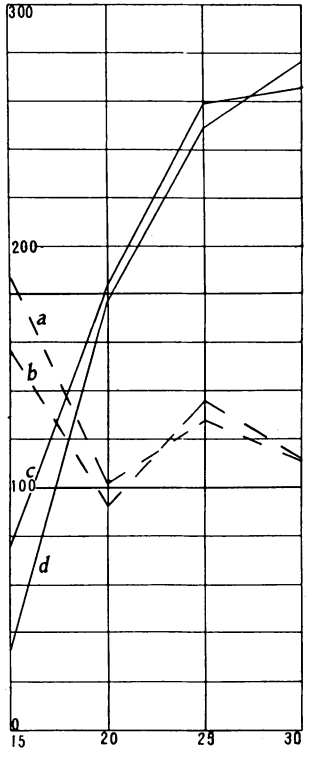
might have been for this reason enough greater than at lower temperatures to cause an appreciable change in the results, and the series was repeated with the hygroscopic conditions more nearly uniform. Charts 12*a* and 11 give the results obtained from the first series, while 11 and 12*b* show the results of the last series. In the first set of experiments the fungus was allowed to grow two weeks, in the last but one week; in the former the cultures for the various temperatures were started at different times, in the latter all were started on the same day and the spores used were from one stock culture.

In charts 11–12*b* the abscissae indicate temperatures as before, but the ordinates express percentages of dry weight instead of percentages of germination. The effect of temperature on the controls is shown in chart 11. In this chart the curves were determined by taking the greatest dry weight as 100 and estimating what per cent. of this the weights secured at other temperatures were. In charts 12*a* and 12*b* these same controls are represented by the ordinates marked 100. In plotting the curves of these two charts, the weight of fungus secured in a given toxic solution at a particular temperature was compared in each case with the weight obtained in the control at the same temperature. The results thus obtained are expressed by the ordinates as mentioned above.

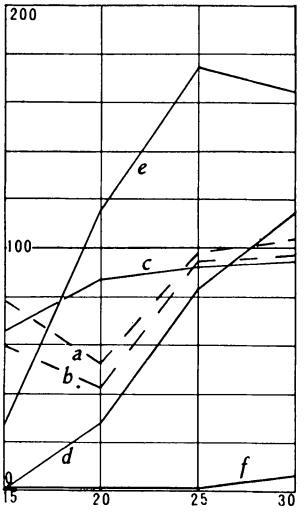
It will be seen that in most instances the curves in the two charts are in close agreement. Where this is not true, as in the case of the controls, the results obtained in the last series should be considered the more reliable for the reason previously given. Taking total growth as a standard, the injurious effects of the toxic agent have decreased with rise of temperature. This decrease is rather to be considered as the result of approaching the optimum for the fungus than as a mere temperature effect. The effects produced by the three chemicals were widely different. The injury resulting in the $CuSO_4$ solutions was not so great, comparatively, at 15° as at 20°. This was true of neither of the acids. Sulfuric acid checked the growth at the lower temperatures, but in no case served as a strong stimulating agent. Nitric acid gave similar injurious effects, but at the higher temperatures served as a remarkable stimulus. It should be remembered that a similar rise at 25° and 30° was obtained in the cell culture curves for HNO_3.



11, Sterigmatocystis. Control curves; *a*, for first series; *b*, for second series.



12*a*, Sterigmatocystis, first series. *a*, *n*/256 $CuSO_4$; *b*, *n*/192 $CuSO_4$; *c*, *n*/32 HNO_3; *d*, *n*/24 HNO_3.



12*b*, Sterigmatocystis, second series. *a*, *n*/128 $CuSO_4$; *b*, *n*/64 $CuSO_4$; *c*, *n*/20 H_2SO_4; *d*, *n*/10 H_2SO_4; *e*, *n*/20 HNO_3; *f*, *n*/10 HNO_3.

The writer hopes by means of further experiments to be able to obtain additional information in regard to the meaning of the difference in action of the chemicals and the significance of the varying effects upon the different fungi. He wishes to acknowledge his indebtedness to Dr. B. M. DUGGAR, Professor of Botany in the University of Missouri, for suggesting the problem reported upon in this paper and for valuable advice throughout the prosecution of the work.

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